THE GGA PROTEINS: ADAPTORS ON THE MOVE

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The GGA proteins are a family of ubiquitously expressed, Arf-dependent clathrin adaptors that mediate the sorting of mannose-6-phosphate receptors between the *trans*-Golgi network and endosomes. Recent studies have elucidated the biochemical and structural bases for the interaction of the GGA proteins with many binding partners, and have shed light on the molecular and cellular mechanisms by which the GGA proteins participate in protein sorting.

PHOSPHOINOSITIDES
Inositol phospholipids that have important roles in signal transduction, and in the recruitment and regulation of peripheral membrane proteins.

ADAPTORS
Proteins that recruit clathrin to membranes and concentrate specific transmembrane proteins in clathrin-coated areas of the membrane.

SORTING SIGNALS
Sequence motifs or structural determinants that interact with specific recognition proteins and determine the trafficking or localization of cellular proteins.

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Protein coats that are associated with the cytosolic face of membranes have key roles in the formation of vesicular transport carriers and in the selection of cargo proteins for incorporation into such carriers[1,2]. The coats are supramolecular assemblies that are deposited on the membranes by the regulated recruitment of their constituent proteins from the cytosol. For many coats, small GTP-binding proteins such as members of the ADP-ribosylation factor (Arf) family (BOX 1), as well as PHOSPHOINOSITIDES, initiate coat assembly by functioning as docking sites for ADAPTOR proteins. The adaptors, in turn, bind: to scaffolding proteins that polymerize to form the outer layer of the coats[1]; to accessory proteins that regulate or affect various aspects of coat function[3]; and to SORTING SIGNALS in cargo transmembrane proteins that mediate their concentration in the coated membrane domains[4].

Coats containing polymerized CLATHRIN as their scaffold protein have been studied in the greatest detail. Two heterotetrameric adaptor-protein (AP) complexes — AP-1 and AP-2 — have long been known to participate in clathrin recruitment to the *trans*-Golgi network (TGN) and endosomes (AP-1), and to the plasma membrane (AP-2). A third AP complex — AP-3 — has also been proposed to function as a clathrin adaptor on endosomes[5], although at least some of its functions are independent of clathrin[6]. These AP complexes are composed of four homologous subunits that are organized into a 'core' domain, two 'ear' or 'appendage' domains, and two long, unstructured 'hinge' segments that connect the ears to the core. The core domains mediate recruitment of the AP complexes to membranes by binding to Arf–GTP (AP-1 and AP-3) and/or phosphoinositides (AP-1 and AP-2). The core domains also bind sorting signals that are contained in the cytosolic tails of cargo transmembrane proteins. The hinge segments of the three AP complexes contain 'clathrin-box' or related sequences that bind to the terminal domain of clathrin. Finally, their ear domains recruit accessory proteins.

For some time, the heterotetrameric AP complexes were regarded as the main, if not the only, adaptors responsible for clathrin-coat assembly and cargo selection. In recent years, however, a growing number of monomeric proteins have been shown to fulfil some or all of the roles of clathrin adaptors (BOX 2). Key to the emergence of this concept of monomeric clathrin adaptors has been the discovery of the Golgi-localized, γ-ear-containing, Arf-binding family of proteins (GGAs). The identification of these proteins by several independent groups was first reported in the year 2000 (REFS 7–10). A rapid succession of studies established the salient features of these proteins, some of which are the basis for the GGA acronym. It soon became clear that the GGAs are ubiquitously expressed, monomeric proteins that: contain a carboxy-terminal domain that is homologous to the carboxy-terminal domain of the γ1- and γ2-adaptin-subunit isoforms of AP-1; are associated with the TGN and endosomes; and interact with Arf proteins, clathrin and the cytosolic tails of intracellular transport receptors. The excitement brought about by these initial studies on the GGAs was expressed in a commentary written by Annette Boman in 2001 (REF. 11). This article ended with the sentence, "Hold on, there is more to come!". Indeed, over

Box 1 | Arf proteins

Arf (ADP-ribosylation factor) proteins are small GTP-binding proteins of the Ras superfamily that have important roles in the regulation of membrane traffic and the actin cytoskeleton. Three classes of Arf proteins have been described, which, in human cells, comprise Arf1 and Arf3 (class I), Arf4 and Arf5 (class II), and Arf6 (class III)[75]. Class I Arfs, in particular, participate in the formation of intracellular transport vesicles and the selection of cargo for incorporation into these vesicles. They do so by recruiting various coat proteins — that is, the GGAs (Golgi-localized, γ-ear-containing, Arf-binding proteins), adaptor protein (AP)-1, AP-3, AP-4, and coatomer protein (COP)I — to membranes, and by activating lipid-modifying enzymes such as phospholipase D1 and type I phosphatidylinositol-4-phosphate 5-kinase, which synthesize phosphatidic acid and phosphatidylinositol-4,5-bisphosphate, respectively. The activity of Arfs is regulated by specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), that convert Arfs to their GTP-bound (active) and GDP-bound (inactive) forms, respectively. Arf-GEFs and Arf-GAPs far outnumber the Arfs and direct their specific activation and inactivation, respectively, at different cellular sites[32].

the past 2 years a wealth of biochemical, structural, morphological and functional information on the GGAs has been obtained. Here, I review this recent information and discuss its implications for the mechanisms of coat-mediated protein sorting in the endosomal–lysosomal system.

Domain organization

There are three GGAs in humans (GGA1, GGA2 and GGA3), two in the yeast *Saccharomyces cerevisiae* (Gga1 and Gga2), and one each in *Caenorhabditis elegans* and *Drosophila melanogaster*. One of the most striking properties of these proteins is their conserved modular organization (FIG. 1). They all consist of a tandem arrangement of three folded domains — designated VHS (Vps27, Hrs, Stam), GAT (GGA and TOM (target of myb)) and GAE (γ-adaptin ear) — separated by two linker sequences. The ~140-residue VHS domain is found in other proteins that are involved in membrane trafficking, such as yeast Vps27 (vacuolar protein sorting 27) and Hse1 (Hbp, Stam and EAST), and their respective mammalian orthologues Hrs (hepatocyte-growth-factor-receptor substrate) and Stam (signal-transducing adaptor molecule). This domain is followed by an ~20-residue linker sequence that is rich in proline residues, which connects it to the next domain, GAT. The ~150-residue GAT domain is found in all GGAs, and shows limited homology to a domain that is found in a protein known as TOM1 (target of myb1) and a related protein known as TOM1L1 (TOM1-like 1) or Srcasm (Src activating and signalling molecule). The next region is the variable 80–286-residue hinge sequence, which does not have significant sequence similarity among the GGAs and is predicted to be largely unstructured. Finally, the 124-residue GAE domain is homologous to the ear domain of the γ1- and γ2-adaptin-subunit isoforms of AP-1 (FIG. 1). This alternation of folded domains and linker sequences gives the GGAs the appearance of 'beads on a string', which is a common feature of many components of protein coats.

Function and structure of the different domains

The modular organization of the GGAs has greatly facilitated the dissection of individual domains for biochemical, structural and functional analyses. Moreover, the isolated domains have proven highly tractable in most *in vitro* and *in vivo* experimental settings. This amenability to experimental manipulation has resulted in a detailed understanding of their properties.

The VHS domain. One reason for the excitement surrounding the GGAs has been the demonstration that the VHS domain of the mammalian proteins functions as a recognition module for sorting signals that are present in the cytosolic tails of sortilin[12] and the two MANNOSE-6-PHOSPHATE RECEPTORS (MPRs) — the cation-independent MPR (CI-MPR) and the cation-dependent MPR (CD-MPR)[13–15]. The MPRs have long been known to sort lysosomal hydrolase precursors to lysosomes as they cycle between the TGN and endosomes[16]. This cycling is dependent on signals known as 'acidic-cluster–dileucine' or 'DXXLL' (where X is any amino acid), which are located near the carboxyl termini of both MPR tails[17–19] (FIG. 2). The key elements of these signals are a cluster of acidic residues, including an essential aspartate residue that is followed three positions downstream by an essential pair of leucine residues. Two further elements that contribute to the overall effectiveness of these signals are a phosphorylatable serine residue that is embedded in the acidic cluster, and the placement of the signal one or two residues from the end of the cytosolic tail. Each of these features is important both for the ability of the MPRs to sort lysosomal hydrolase precursors *in vivo*[17–20] and for interactions with the GGA VHS domains in yeast two-hybrid and *in vitro* binding assays[13–15,20,21]. This correlation of sorting and binding data supports the notion that the GGAs function as adaptors for the sorting of MPRs between the TGN and endosomes.

In addition to the MPRs, several other transmembrane proteins possess DXXLL signals that interact with the VHS domain of the GGAs (FIG. 2). Although little is known about the trafficking of these transmembrane proteins, a reasonable inference is that they might also cycle between the TGN and endosomes. Strikingly, the hinge segments of GGA1 and GGA3 each contains an internal DXXLL sequence that interacts with the ligand-binding site on the corresponding VHS domain[22]. Phosphorylation by CASEIN KINASE 2 (CK2) of a serine residue located three residues upstream of the internal DXXLL sequence modulates the interaction with the VHS domain[23]. This intrinsic DXXLL sequence is thought to have an autoinhibitory role that is crucial for the function of the GGAs *in vivo*[24] (see below). In contrast to the GGAs, the proteins TOM1, TOM1L1, Hrs and Stam have VHS domains that do not bind DXXLL signals[13,15]. Finally, the GGA VHS domains do not bind another type of dileucine-based sorting signal that is defined by the motif [DE]XXXL[LI] (either of the residues in the square brackets is allowed at the indicated positions)[2,13]. These findings emphasize the specificity of GGA VHS–DXXLL-signal interactions.

The structural basis for the specific recognition of DXXLL signals by the GGA VHS domains has been elucidated by X-ray crystallography[25–27]. The VHS domain

CLATHRIN
A structural protein that is composed of three heavy chains and three light chains. An amino-terminal globular domain known as the 'terminal domain' binds adaptor proteins. Clathrin polymerizes into polyhedral lattices to form the scaffold of membrane coats.

MANNOSE-6-PHOSPHATE RECEPTORS
Type I integral membrane proteins that mediate the sorting of newly synthesized lysosomal hydrolase precursors from the *trans*-Golgi network to endosomes *en route* to lysosomes. Sorting is mediated by the recognition of mannose-6-phosphate groups on the lysosomal hydrolase precursors by the lumenal domain of the mannose-6-phosphate receptors.

CASEIN KINASE 2
A ubiquitous and constitutively active protein kinase that phosphorylates serine or threonine residues that are embedded in acidic sequences fitting the consensus [ST]XX[DEpSpT] (where X is any amino acid, pS is phosphoserine and pT is phosphothreonine; any of the residues in the square brackets are allowed at the indicated positions).

Box 2 | Monomeric clathrin adaptors

In addition to the heterotetrameric adaptor-protein (AP) complexes AP-1, AP-2 and AP-3, many monomeric proteins have been recently shown to function as clathrin adaptors. These include: the GGAs (Golgi-localized, γ-ear-containing, Arf (ADP-ribosylation factor)-binding proteins) and enthoprotin/epsinR[56–59] at the *trans*-Golgi network; epsin 1 (Eps15-interacting)[76,77], ARH (autosomal recessive form of hypercholesterolaemia)[78,79], Dab2 (Disabled 2)[80,81], HIP1 (Huntingtin-interacting protein 1)[82], HIP1R (HIP1-related)[83] and Numb[84] at the plasma membrane; and Hrs (hepatocyte-growth-factor-receptor substrate)[85,77] and Stam (signal-transducing adaptor molecule)[86] on endosomes. These proteins: are recruited to membranes by virtue of interactions with Arfs or phosphoinositides; interact with clathrin through 'clathrin-box' or related peptide sequences; bind to AP-1 or AP-2 or to one another through specific motifs; and recognize peptide signals in, or ubiquitin conjugated to, the cytosolic domains of transmembrane proteins. The ability of some of these proteins to interact with the 'ear' domains of AP-1, AP-2 or the GGAs has led to them being included among the class of 'accessory proteins'. The discovery of these monomeric adaptors has led to a reassessment of the structure of clathrin coats, which are now viewed as scaffolds that support a diverse array of recognition proteins for different transmembrane cargoes.

of the GGAs consists of a right-handed superhelix of eight α-helices, which is similar to the VHS domains of TOM1 (REF. 28) and Hrs[29], and reminiscent of the ENTH (epsin amino-terminal homology) domain of the epsins[30] and the ANTH (AP180 (assembly protein of 180 kDa) amino-terminal homology) domain of both AP180 and CALM (clathrin assembly lymphoid myeloid leukaemia)[31] (FIG. 3a). The epsins and AP180/CALM constitute two other families of monomeric coat proteins with characteristics of adaptors. A DXXLL peptide binds in an extended conformation to a cleft between the sixth and eighth α-helices of the GGA VHS domain[25,26]. The key D and LL residues of the signal bind to an electropositive pocket and two shallow hydrophobic pockets, respectively, on the surface of the GGA VHS domain, whereas the phosphoserine residue[21] and the terminal carboxyl group[25] contribute ancillary electrostatic interactions[25] (FIG. 3a). The residues of the mammalian GGA VHS domains that are involved in interactions with DXXLL signals are not conserved in the VHS domains of TOM1, TOM1L1, Hrs, Stam and the yeast Ggas, which explains why these proteins are unable to bind such signals[25,26].

RAB4/RAB5
Rab proteins are monomeric small GTPases that, along with their effectors, mediate the tethering of membrane vesicles to the actin cytoskeleton and to acceptor organelles.

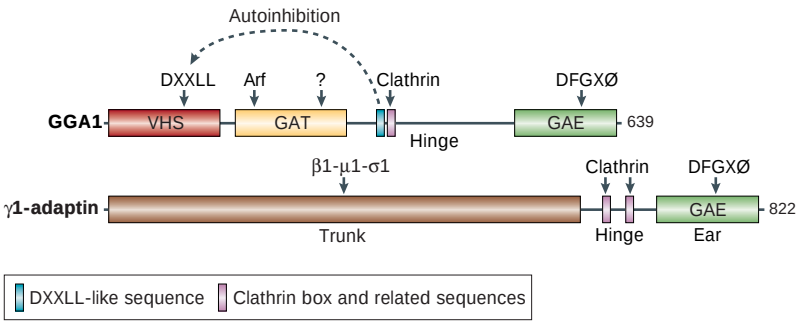

Figure 1 | **Schematic representation of the domain organization of GGA1 and γ1-adaptin.** The structure of GGA1 (Golgi-localized, γ-ear-containing, Arf (ADP-ribosylation factor)-binding protein 1) is representative of that of other GGAs, and the structure of γ1-adaptin is similar to that of γ2-adaptin (γ1-adaptin and γ2-adaptin are isoforms of one subunit of the heterotetrameric (γ–β1–μ1–σ1) adaptor-protein (AP)-1 complex). The number of amino-acid residues in each protein is indicated on the right. The sequences or proteins that bind to each domain are indicated by arrows. The VHS (Vps27, Hrs, Stam) domain binds DXXLL-type sequences (where X is any amino acid) similar to those shown in FIG. 2, including an autoinhibitory DXXLL sequence in the hinge segment of the GGAs. The GAT (GGA and TOM (target of myb)) domain has two binding sites, one for Arf and the other for an unidentified protein that is indicated by a question mark. The hinge segments of both GGA1 and γ1-adaptin contain variants of the clathrin-box motif (LLDDE in GGA1; there are two copies of LLDLL in γ1-adaptin). The GAE (γ-adaptin ear) domains bind DFGXØ-type sequences (where Ø is a bulky hydrophobic residue) similar to those listed in FIG. 5. The trunk domain of γ1-adaptin interacts with the other three subunits of the AP-1 complex.

The GAT domain. Among the first properties of the GGAs to be discovered was their ability to interact with the GTP-bound, but not the GDP-bound, forms of Arf1 (REF. 8) and Arf3 (REF. 7). These two Arfs are closely related in primary structure and belong to the 'class I' group of Arfs, which control the recruitment of other coat proteins such as coatomer protein (COP)I, AP-1, AP-3 and AP-4 to membranes[32] (BOX 1). Molecular dissection of the GGAs showed that the ability to bind Arf proteins resides in their GAT domains[8,33–35]. Mutational analyses defined a region near the amino terminus of the GAT domain that contains key residues for interaction with Arf[33]. Mutation of these residues abrogated not only binding to Arf, but also recruitment of the full-length mammalian GGAs to the TGN *in vivo*[33,34]. In addition, fusion of the GGA GAT domain to green fluorescent protein (GFP) resulted in a chimaera that was efficiently targeted to the TGN[8,34]. These observations indicate that, at least in mammalian cells, the interaction of the GAT domain with Arf is both necessary and sufficient for the recruitment of the GGAs to the TGN. However, the deposition of the GFP–GAT chimaera on the TGN seems to be homogeneous[8] in contrast to the punctate distribution of endogenous GGAs[7–9], which points to a possible role for the other GGA domains in specifying localization to discrete sites in the TGN. In this regard, the VHS and GAE domains of the *S. cerevisiae* Ggas have been shown to cooperate with the GAT domain to direct the localization of the Ggas to the late Golgi complex[34].

The GAT domains of mammalian GGA1 and GGA2 have also been shown to interact directly with Rabaptin-5 (REF. 36) — a RAB4/RAB5 effector that is present in a complex with the Rab5 guanine nucleotide exchange factor (GEF) Rabex-5 (REF. 37). Rabaptin-5 is predicted to consist of two mainly α-helical domains (possibly structured as coiled-coils) that are connected by a long, unstructured sequence (FIG. 4). Mutational analyses have delineated the regions involved in the GAT–Rabaptin-5 interaction to a segment towards the carboxyl terminus of the GAT domain and a predicted coiled-coil segment in the carboxy-terminal folded domain of Rabaptin-5 (REF. 36). Given that the Rabaptin-5–Rabex-5 complex has been previously implicated in endosomal tethering/fusion events that enable the transfer of materials between endosomes[37], the interaction of this complex with the GGAs might point to its possible involvement in cargo transfer from TGN-derived coated carriers to endosomes.

The recent resolution of the crystal structure of the GAT domain of the GGAs has shed light on the nature of the interactions that link the GAT domain to its

CI-MPR	Tm-151-SFHDDSDEDLLHI
CD-MPR	Tm-54-EESEERDDHLLPM
Sortilin	Tm-41-GYHDDSDEDLLE
SorLA	Tm-41-ITGFSDDVPMVIA
LRP3	Tm-237-MLEASDDEALLVC
β-secretase	Tm-9-HDDFADDISLLK
GGA1	350-ASVSLLDDELMSL-275
GGA3	384-NALSWLDEELLCL-326

Figure 2 | **Proteins with DXXLL-type sorting signals that bind to the VHS domain of the GGAs.** The figure shows transmembrane proteins that contain DXXLL-type signals in their cytosolic tails and GGAs (Golgi-localized, γ-ear-containing, Arf (ADP-ribosylation factor)-binding proteins) that contain internal DXXLL-type sequences in their hinge segments. The two crucial leucine or bulky hydrophobic residues are shown in red, the crucial aspartate is shown in blue, and serine residues that are potential targets for phosphorylation are shown in green. The position of the transmembrane domain (Tm) and the number of residues before and/or after the signals is indicated. CD-MPR, cation-dependent mannose-6-phosphate receptor; CI-MPR, cation-independent mannose-6-phosphate receptor; LRP3, low-density-lipoprotein-receptor-related protein 3; SorLA, sorting-protein-related receptor containing low-density-lipoprotein-receptor class A repeats; VHS, 'Vps27, Hrs and Stam'. For further information, please see references as follows: CI-MPR[13–15]; CD-MPR[13,20]; Sortilin[12,15]; SorLA[87]; LRP3 (REF. 15); β-secretase[88]; GGA1 and GGA3 (REF. 22).

binding partners. The GAT domain consists of an elongated, all α-helical fold that comprises two subdomains — an amino-terminal 'hook' that binds Arf and a carboxy-terminal 'triple-α-helical bundle' that binds Rabaptin-5 (REFS 38–41; FIG. 3b). The hook subdomain is a helix–loop–helix structure that interacts through hydrophobic and hydrogen-bond interactions with the switch 1 and switch 2 regions of Arf–GTP[39] (FIG. 3b). These are precisely the regions of Arf that undergo large conformational changes on occupancy by GTP or GDP[42]. The structure of the hook subdomain is unique and is not found in other Arf-binding modules, such as the Arf-GEF and Arf-GAP (GTPase-activating protein) domains of known structure[39]. Indeed, the manner by which each of these modules interacts with Arf is distinct. Nevertheless, the surfaces on Arf to which they bind overlap significantly. For example, the Arf-GAP domain contacts switch 2 (REF. 43), whereas the Arf-GEF domain contacts both switch 1 and switch 2 (REF. 44). Therefore, the interactions of these three Arf-binding modules with Arf must be mutually exclusive, which explains why the GAT domain inhibits the action of Arf-GAP on Arf[33,45].

The second α-helix of the hook subdomain extends further than the first and, with other α-helices, forms an up-and-down bundle of three α-helices[38–41] (FIG. 3b). Interestingly, theoretical analyses of the surface of this helical-bundle subdomain predict the existence of a conserved cluster of hydrophobic amino-acid side chains, which might constitute a binding site for another protein[38,40,41]. This domain resembles that of the amino-terminal domain of some target-membrane (t)-SNARES, including syntaxin 1a, syntaxin 6, Sso1 (suppressor of sec one 1) and Vam3 (vacuolar morphology 3)[38,40]. This resemblance extends even to the presence of the conserved hydrophobic patch, which in the case of these t-SNAREs is involved in intramolecular, regulatory interactions with 'SNARE motifs' that are located towards the carboxyl terminus of the proteins[46]. It will be interesting to determine whether the hydrophobic patch in the helical-bundle subdomain of the GGA GAT is a binding site for Rabaptin-5 or a SNARE. Either of these possibilities could implicate the GGAs in a vesicle-tethering or fusion event. This hydrophobic patch is 35-Å away from the Arf-binding site, which means that Arf and a putative second partner could bind to the GAT domain simultaneously.

The hinge segment. The hinge segment of the GGAs consists of a long polypeptide sequence that is predicted to be largely devoid of secondary structure. If fully extended, this sequence could reach upwards of 300 Å. In other coat proteins, sequences of this kind harbour short peptide motifs that engage specific peptide-recognition modules. For example, the hinge segments of the β1 subunit of AP-1, the β2 subunit of AP-2 and the β3 subunit of AP-3 all contain clathrin-box motifs, which fit the L[LI][DEN][LF][DE] consensus sequence (any residue in the square brackets is allowed at the indicated positions) and interact with the terminal domain of the clathrin heavy chain[5,47]. The hinge segments of the three human GGAs and the two *S. cerevisiae* Ggas contain variants of the clathrin-box motif that allow them to bind clathrin *in vitro*[14,33,48,49]. Moreover, overexpression of the GGAs *in vivo* causes increased recruitment of clathrin to the TGN[33]. These observations, together with the colocalization of GGAs and clathrin in the area of the TGN and endosomes[33,50,51], as well as the genetic interactions of the Ggas with clathrin in yeast[48], support the notion that the GGAs function in association with clathrin.

As mentioned above, the GGA1 and GGA3 hinge segments also contain internal DXXLL-like sequences that bind to their corresponding VHS domains on CK2-mediated phosphorylation of an adjacent serine residue[22,23]. In addition, the hinge segments of the GGAs bind to the ear domain of γ1-adaptin[24], although the exact sequences involved and the mode of interaction have not been determined. This observation indicates that the GGAs and AP-1 interact with one another and might therefore cooperate in sorting cargo at the TGN[24].

The GAE domain. The GAE domains of the GGAs and the related ear domains of the γ1- and γ2-adaptin-subunit isoforms of AP-1 have been found to interact with a specific cohort of accessory proteins in yeast two-hybrid and *in vitro* binding assays. This cohort includes γ-synergin[9,52], p56 (REF. 53), Rabaptin-5 (REFS 9,36,54,55) and an epsin-related protein that is known as enthoprotin[56], epsinR[57,58] or CLINT (clathrin interacting protein localized in the *trans*-Golgi region)[59] in mammals, and two epsin-related proteins known as Ent3 and Ent5 in *S. cerevisiae*[60] (FIG. 4). Although all of these proteins interact with the GGAs *in vitro*, the GGAs and γ-adaptins show distinct interaction preferences *in vivo*. For example,

SNARES (soluble *N*-ethyl-maleimide-sensitive attachment protein receptors). Highly α-helical proteins that mediate the specific fusion of vesicles with target membranes. SNAREs have been classified into two complementary classes that are referred to as vesicle-membrane SNAREs (v-SNAREs) and target-membrane SNAREs (t-SNAREs).

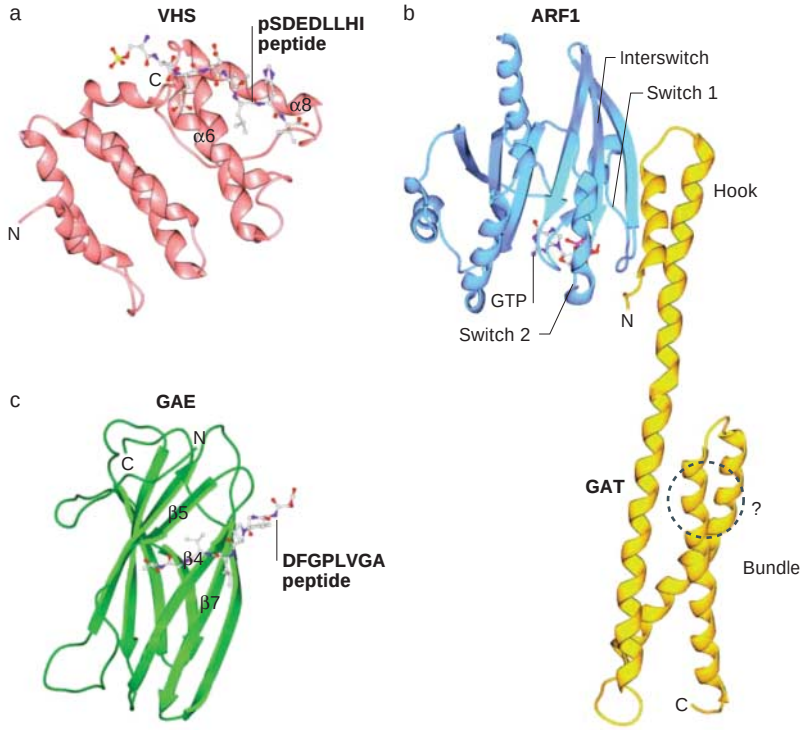

Figure 3 | **Crystal structures of the different domains of the GGAs. a** | Crystal structure of the VHS (Vps27, Hrs, Stam) domain of GGA3 (Golgi-localized, γ-ear-containing, Arf (ADP-ribosylation factor)-binding protein 3) in a complex with a phosphorylated peptide (pSDEDLLHI, where pS indicates phosphoserine) from the cation-independent mannose-6-phosphate receptor (Protein Data Bank (PDB) accession code 1LF8)[21]. **b** | Crystal structure of the GAT (GGA and TOM (target of myb)) domain of GGA1 in a complex with the GTP-bound form of ARF1 Q71L that lacks its amino-terminal 17 residues (PDB accession code 1J2H for the human GGA1 GAT domain, and 1J2J for the ARF1(Δ17–Q71L)–GAT-domain complex)[39]. The dashed circle with a question mark on the GAT domain indicates a putative hydrophobic binding site for another protein. **c** | Crystal structure of the GAE (γ-adaptin ear) domain of GGA3 in a complex with a DFGPLVGA peptide from the accessory protein Rabaptin-5 (PDB accession code 1P4U)[63]. The amino (N) and carboxyl (C) termini of the domains are indicated. Please refer to the text for further information.

the GGAs interact preferentially with p56, whereas AP-1 tends to interact with γ-synergin in cells[53]. It has recently been shown that all of these proteins share a canonical peptide motif — DFGXØ (where Ø is a bulky hydrophobic residue) — that mediates their interactions with the GAE domains[36,58,60] (FIG. 5). This motif is generally found in unstructured regions of the accessory proteins, which in the case of Rabaptin-5 corresponds to the linker between the two folded, α-helical domains[36] (FIG. 4). As mentioned above, the GGAs also interact with the carboxy-terminal α-helical domain of Rabaptin-5 through their GAT domain, which indicates that these interactions are divalent[36]. In addition to one or more DFGXØ motifs, all of the GAE-binding partners described so far have modular domains and/or other interaction motifs that are characteristic of components of the trafficking machinery. This contributes to the propagation of a network of interacting proteins that is required to form, and target, vesicles coated with GGAs and/or AP-1.

Whereas the functions of γ-synergin and p56 are unknown, some functional information is available for Rabaptin-5 and the epsin-related proteins. As mentioned above, Rabaptin-5 participates, as part of a complex with Rabex-5, in endosomal tethering/fusion events[37]. Enthoprotin, Ent3 and Ent5 have an amino-terminal ENTH domain[56–60] similar to that found at the amino termini of epsins 1, 2 and 3 (REF. 30). The epsin 1 ENTH domain binds phosphatidylinositol-4,5-bisphosphate in such a way that it becomes partially buried in the cytosolic leaflet of the plasma membrane[61]. This induces curvature of the plasma membrane and contributes to the formation of clathrin-coated buds. The ENTH domain of enthoprotin, on the other hand, binds phosphatidylinositol-4-phosphate[57,58] — a phosphoinositide that is enriched in TGN membranes. It is therefore possible that enthoprotin could have a role in membrane bending at the TGN, which is similar to that of epsin 1 at the plasma membrane. In addition to DFGXØ motifs, enthoprotin and Ent5 also have canonical clathrin-binding motifs[56–60], which might contribute to their recruitment to clathrin coats or to the assembly of such coats.

The crystal structures of the GAE domains of mammalian GGA1 and GGA3 have been solved[53,62,63] and were found to be similar to that of the ear domain of γ1-adaptin[64,65] (FIG. 3c). The GAE domain consists of an eight-stranded β-sandwich that is made up of a five-stranded β-sheet and a three-stranded β-sheet. A DFGXØ-containing peptide from Rabaptin-5 (REF. 63) or p56 (REF. 62) binds in an extended conformation to a surface that is formed by β-strands 4, 5 and 7 (FIG. 3c). The two anchoring residues of these peptides are the phenylalanine and the bulky hydrophobic residue, each of which binds to a hydrophobic pocket on the surface of the GAE domain. The aspartate residue and other residues adjacent to the motif add weak electrostatic and hydrophobic interactions that contribute to the overall strength and fine specificity of the interactions. The glycine residue does not interact with the GAE domain, but allows the correct positioning of the two hydrophobic anchoring residues. The peptide-binding residues on the GAE domain are conserved in all the GGAs and γ-adaptins[62,63]. Differences in the exact sequence of the peptide motifs and the shape of the binding site could account for the distinct binding preferences that are shown by the GGAs and γ-adaptins for specific accessory proteins.

The GAE domain of GGA1 also interacts with clathrin[33], although the biochemical and structural bases for this interaction have not been determined.

Resemblance to AP complexes. The combined properties of all the GGA domains are consistent with the notion that the GGAs function as Arf-dependent clathrin adaptors for the sorting of MPRs and other transmembrane cargo at the TGN. It is remarkable that the several interactions that are required for this function are all restricted to the single polypeptide chain of the GGAs, whereas similar interactions are divided among the subunits of the heterotetrameric AP complexes. So, the GGAs can be thought of as 'compact' adaptors. Despite the differences in quaternary

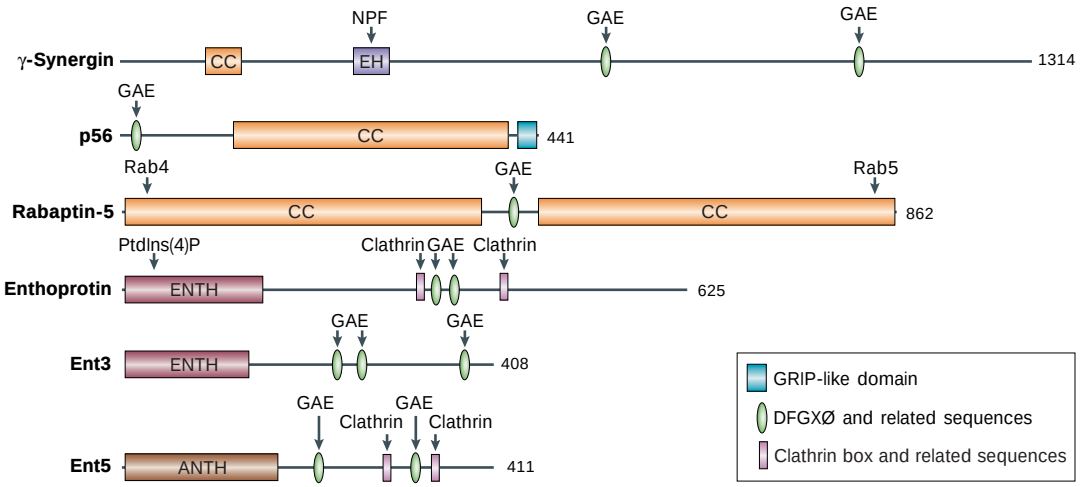

Figure 4 | **Schematic representation of the structures of GAE-binding accessory proteins.** The figure shows the domain organization of accessory proteins that bind to the GAE (γ-adaptin ear) domain of the GGAs (Golgi-localized, γ-ear-containing, Arf (ADP-ribosylation factor)-binding proteins) and/or the ear domain of the γ-adaptins. The domains and peptide motifs of the accessory proteins are highlighted by coloured boxes and ovals, and other proteins, domains or peptide motifs that bind to the accessory proteins are indicated by arrows. The accessory-protein domains are CC (coiled-coil), EH (Eps15-homology), GRIP-like (domain found in golgin-97, RanBP2α, Imh1 and p230/golgin-245), ENTH (epsin amino-terminal homology) and ANTH (AP180 amino-terminal homology). The EH domain binds NPF motifs. Also highlighted in the accessory proteins are GAE-binding, DFGXØ-type sequences (where Ø is a bulky hydrophobic residue) similar to those listed in FIG. 5, and clathrin-binding sequences. Rabaptin-5 is part of a complex with Rabex-5, but the site of Rabex-5 binding on Rabaptin-5 is not known. The number of amino-acid residues in each protein is indicated on the right. PtdIns(4)P, phosphatidylinositol-4-phosphate. Please refer to the text for details on the accessory proteins.

structure, the GGAs and AP complexes show a marked structural resemblance. Both types of adaptor have: predominantly α-helical amino-terminal domains (VHS–GAT and core) that bind to sorting signals and docking factors; long flexible segments with peptide motifs for binding to clathrin; and predominantly β-sheet ear domains that bind accessory proteins. The flexibility and length of the hinge segments allow the peptide motifs and ear domains to extend from the membrane to the clathrin lattice and beyond, as well as to fold back towards the space near the membrane where interdomain interactions take place.

Assembly and disassembly of GGA coats

On the basis of the properties of the GGA domains, the outline of a mechanism for the assembly of GGA-containing coats has begun to emerge (FIG. 6). Although much remains to be learned about the interplay among all the coat components and the order in which each functions, this mechanism probably involves the steps described below.

Membrane recruitment. As is the case for other adaptors, localized activation of Arf probably initiates the recruitment of the GGAs to the TGN. An Arf-GEF converts Arf–GDP to Arf–GTP, which results in the exposure of a myristoylated amino-terminal α-helix that tethers Arf–GTP to the membrane, as well as a structural rearrangement of the switch 1 and switch 2 regions that allows the binding of Arf effectors[32]. Arf–GTP therefore becomes a docking protein for the recruitment of the GGAs to membranes through interaction of the Arf–GTP switch and interswitch region with the hook subdomain of the GAT domain[39] (FIG. 3b). Given that Arf–GTP also associates with the *cis*-Golgi cisternae, the recruitment of the GGAs to the TGN must be specified by some other protein or lipid. Possible candidates for this other docking factor are the Arf-GEFs or Arf-GAPs themselves, or a particular phosphoinositide that is enriched in TGN membranes. Binding of the GAT domain to Arf–GTP hinders the action of the Arf-GAPs, which produces a transient stabilization of the GGA–Arf–GTP complex[33,45] that might be a requisite for subsequent assembly steps.

Signal recognition. The binding of the GAT domain to Arf places the VHS domain in close proximity to the membrane, where it can interact with DXXLL-type signals in the cytosolic tails of the MPRs and other cargo transmembrane proteins (FIG. 2). This interaction is a target for various regulatory inputs. First, cytosolic GGA1 and GGA3 are present in an autoinhibited state, owing to the CK2-phosphoregulated binding of an internal DXXLL-like sequence to the VHS domain[22]. Release from autoinhibition requires dephosphorylation by a protein phosphatase 2A (PP2A)-like enzyme, which might occur after binding of the GGAs to Arf–GTP[23]. Second, CK2-mediated phosphorylation of a serine residue upstream of the DXXLL signals that are situated in the transmembrane cargo tails results in tighter binding to the VHS domain[21]. As the effects of these two phosphoregulatory events are counterposed, they must be subject to spatial or temporal control, or

be carried out by different kinases. Third, the GAT domain has to be released from Arf–GTP for the VHS domain to bind to the signals, in what has been likened to a 'hand-off' of the GGA from Arf to the transmembrane cargo tail[66].

Clathrin assembly. The membrane-bound GGAs contribute to the recruitment of clathrin to the TGN by virtue of interactions between clathrin-box-like sequences in the hinge segment of the GGAs and the terminal domain of the clathrin heavy chain[14,33,49]. The GAE domain of GGA1 adds to the avidity of interactions with clathrin by binding to a still undetermined site on clathrin. At present, it is unclear whether the GGAs alone can assemble clathrin on the TGN or whether they do so in cooperation with other clathrin-binding proteins such as AP-1 and enthoprotin/Ent5.

Binding of accessory proteins. Finally, the GAE domain of the GGAs and the ear domain of the γ-adaptin subunit of AP-1 function as platforms for the assembly of a network of accessory proteins that mediate various functions of the coats (FIG. 4). Enthoprotin might curve the membrane to allow vesicle budding and perhaps sort other cargo into the forming carriers. The Rabaptin-5–Rabex-5 complex could mediate interactions with Rab5 or Rab4 in the process of targeting the GGA-containing carriers to endosomes. The EH (Eps15 homology) domain of γ-synergin could be used to engage proteins containing NPF motifs, and p56 could interact with other proteins through its GRIP-like domain (domain found in golgin-97, RanBP2α, Imh1 and p230/golgin-245; FIG. 4). These accessory proteins could mediate or regulate coat assembly and disassembly, vesicle budding, interactions with the cytoskeleton and vesicle targeting or fusion.

γ-synergin	664-SLAD**DFGE**E**F**SLFGE-636
p56	MDDD**DFGG**F**E**AAET-427
Rabaptin-5	437-LDES**DFGP**L**V**GADS-415
Enthoprotin	366-SGNG**DFGD**W**S**AFNQ-245
Ent3	373-DDDD**EFGE**M**H**GGAV-21
Ent5	325-DDDD**EFGD**F**Q**SETS-72

Figure 5 | **GAE-binding motifs in accessory proteins.** The figure shows examples of putative GAE (γ-adaptin ear)-binding sequences that have been inferred from binding and crystallographic analyses. The residues that conform to the DFGXØ motif (where X is any amino acid and Ø is a bulky hydrophobic residue) are indicated in red. Not all of these residues have been shown to be required for binding, and other neighbouring residues might also contribute to the interaction. Other variants of this motif might also be present in the accessory proteins. The numbers shown indicate the number of residues that are found before and after the listed sequences. For further information, please see references as follows: γ-synergin[52]; p56 (REFS 53,38); Rabaptin-5 (REFS 36,63); Enthoprotin[58]; and Ent3 and Ent5 (REF. 60).

GGA-containing carriers

The GGA-dependent sorting process would be expected to result in clathrin-coated vesicles (CCVs) that contain GGAs as their adaptors and MPRs as their cargo. Indeed, coated buds and vesicles containing GGAs together with clathrin, AP-1 and MPRs have been visualized in the area of the TGN by immunoelectron microscopy[24,33,50]. However, the GGAs are not enriched in purified CCVs[9]. This could be due to dissociation of the GGAs during preparation of the CCVs, as indicated by the lability of GGA association with membranes on permeabilization[51]. Alternatively, the GGAs might have only an intermediary role in packaging cargo into forming vesicles. In this regard, a transfer of MPRs from the GGAs to AP-1 has been proposed to occur on phosphorylation of GGA1 and GGA3 by a CK2-like enzyme that is associated with AP-1 (REF. 24). Phosphorylation would return these GGAs to their autoinhibited state, and dissociation of the GGAs from membranes would ensue. Eventually, the CCVs would lose their coat proteins *en masse*, after which the uncoated remnants would fuse with neighbouring endosomes to deliver their cargo.

Recent studies have provided tantalizing clues to the existence of another type of GGA-containing carrier that is derived from the TGN. Imaging proteins labelled with variants of GFP in live cells has highlighted the existence of a population of tubular–vesicular carriers that contain GGA1 (REFS 13,50; see Movie 1 online), AP-1 (REFS 50,67,68), clathrin[50], enthoprotin/epsinR[57] and MPRs[13,68]. These carriers are more pleiomorphic, larger and longer-lived than conventional CCVs. They move along microtubules from the area of the TGN to the peripheral cytoplasm, where they engage in interactions with endosomes[50,68]. The carriers are probably part of a system that enables the long-range distribution of cargo to outlying areas of the cytoplasm. Strikingly, the coat proteins on these carriers often persist until they reach the cell periphery, which makes it possible for the coats to have post-budding roles in the recruitment of motor molecules[69] and tethering/fusion factors[36,55].

Function in sorting to the lysosome/vacuole

The most reasonable interpretation of all the biochemical, structural and morphological evidence is that the mammalian GGAs have roles in packaging MPRs and their ligands (that is, lysosomal hydrolase precursors) into CCVs or clathrin-coated carriers that bud from the TGN and deliver their cargo to either early or late endosomes. The evidence for this function, however, is still largely indirect. The only reported attempts to link the GGAs with sorting of the MPRs *in vivo* involved the use of a truncated GGA1 construct that consisted of just the VHS and GAT domains[13,33]. Moderate overexpression of this construct in cells caused the accumulation of CI-MPR and CD-MPR at the TGN and their depletion from peripheral endosomes, which probably occurred because of interference with the incorporation of the receptors into clathrin-coated buds. Although this finding demonstrates a functional interaction of GGA1 with MPRs *in vivo*, it does not rule out the possibility that the GGAs could have roles elsewhere, other than at the TGN. In fact, a substantial amount of the GGAs seem to exist in association with peripherally distributed structures[8,50],

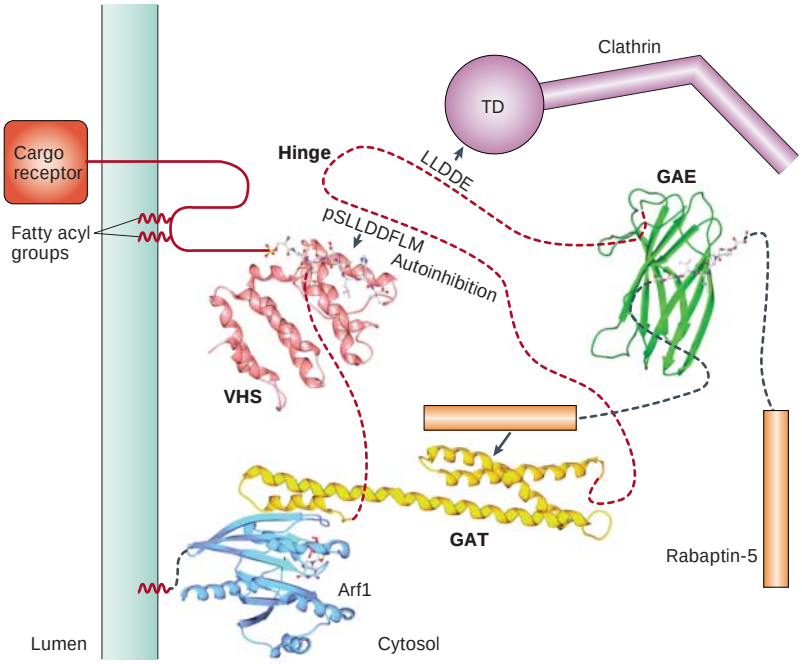

Figure 6 | **Schematic representation of the assembly of GGA-containing coats.** Membrane-tethered Arf (ADP-ribosylation factor)–GTP (blue) binds to the GAT (GGA and TOM (target of myb); yellow) domain, which results in the recruitment of the GGA (Golgi-localized, γ-ear-containing, Arf-binding protein) to the membrane. The VHS (Vps27, Hrs, Stam; pink) domain binds DXXLL-type signals (where X is any amino acid) in the tails of mannose-6-phosphate receptors and other transmembrane cargo. An autoinhibitory, internal DXXLL sequence (that is, pSLLDDFLM in GGA1, where pS indicates phosphoserine) in the hinge segment regulates signal recognition. The hinge segment binds through clathrin-box-like sequences (for example, LLDDE in GGA1) to the terminal domain (TD) of clathrin, and the GAE domain (γ-adaptin ear; green) binds through DFGXØ-like sequences (where Ø is a bulky hydrophobic residue) to accessory proteins (Rabaptin-5 is shown in this figure, which also interacts with the GGA GAT domain). The position of the Rabaptin-5 binding partner Rabex-5 is not known and is therefore not shown in the figure. The order of these different steps has not been established. Other proteins such as adaptor protein (AP)-1 and enthoprotin/Ent5 might intercalate into these coats and might also participate in the recruitment of clathrin, cargo and accessory proteins. The red dashed lines represent the unstructured sequences in the GGA, and the black dashed lines represent the unstructured sequences in Rabaptin-5.

where they could also function in protein sorting. It is unclear at present if this represents continued shepherding of the MPRs as they travel along the TGN–endosomal pathway or the performance of an entirely different sorting function at endosomes.

Considerably more is known about the physiological roles of the Ggas in *S. cerevisiae*. Deletion of either of the genes encoding Gga1 and Gga2 results in negligible phenotypic changes[8,9,48,70]. However, the combined deletion of both genes impairs proteolytic processing of the inactive precursors of the vacuolar hydrolases carboxypeptidase Y (CPY), proteinase A (PrA) and carboxypeptidase S (CPS)[8,9,48,70]. As processing to produce the active forms of the enzymes normally occurs in the vacuole, this phenotype is indicative of defective transport to the vacuole. Soluble pro-CPY and pro-PrA are sorted from the late Golgi (equivalent to the mammalian TGN) to endosomes by binding to Vps10 (REF. 71), a transmembrane receptor that functions in a manner analogous to MPRs. Pro-CPS is a transmembrane protein that is sorted from the late Golgi complex to endosomes in a Vps10-independent manner. In late endosomes, also known as the prevacuolar compartment in yeast, pro-CPS undergoes transport into membrane invaginations by a process that is dependent on ubiquitylation of the pro-CPS tail[72,73]. The pro-CPS sorting defect is more severe than the pro-CPY and pro-PrA sorting defects in *gga1*Δ*gga2*Δ cells[48], which indicates that the sorting of pro-CPS is more strictly dependent on the Ggas.

The sorting of pro-CPY, pro-PrA and pro-CPS involves several steps, and it is unclear at present which one is compromised by the absence of the Ggas. Studies on the late endosomal SNARE Pep12, however, have shown that this protein is missorted from the Golgi to early endosomes in *gga1*Δ*gga2*Δ cells, which implies that the Ggas are involved in transport from the late Golgi to late endosomes[74]. In line with this observation, *gga1*Δ*gga2*Δ cells secrete an aberrantly-cleaved, 'pseudo-mature' form of CPY[8,9], which indicates that pro-CPY is mistargeted to another proteolytically-active compartment before release into the periplasmic space. Finally, the proteolytic processing of the mating pheromone precursor pro-α-factor is also impaired in *gga1*Δ*gga2*Δ cells[48,49]. Pro-α-factor is normally processed by the Kex2 endopeptidase, a transmembrane enzyme that cycles between the late Golgi and late endosomes. In the absence of the Ggas, Kex2 seems unable to travel to a late endosomal compartment from where it can be retrieved back to the late Golgi. Instead, it follows a circuitous route through the plasma membrane to the vacuole, where it is degraded[48,49]. On the basis of these observations, the yeast Ggas would be expected to interact with the cytosolic tails of Vps10, pro-CPS, Pep12 and/or Kex2. However, no such interactions have been reported so far. In addition, these yeast transmembrane proteins do not have canonical DXXLL signals, nor do the VHS domains of the yeast Ggas have the residues that are involved in the recognition of such signals[25]. It is therefore probable that the yeast Ggas recognize a sorting determinant that is distinct from DXXLL signals. Another important difference is that the yeast Ggas seem less dependent on Arf and clathrin binding for function as compared with their mammalian counterparts[34,49]. Taken together, these studies indicate that the yeast Ggas are involved in the sorting of transmembrane proteins to the vacuole, although their exact site and mechanism of action remain to be determined.

Perspectives

In just over 3 years, the GGAs have moved to the fore in studies of the mechanisms of coat-mediated protein sorting at the TGN. Rapid progress has occurred in two main areas — obtaining biochemical and structural data on the GGAs and phenotypic data on Gga-deficient *S. cerevisiae* strains. So, we now have detailed descriptions of the protein interactions that enable the GGAs to function as sorting adaptors, and of the ultimate consequences of their deficiency in yeast. However,

a significant knowledge gap remains between these molecular and physiological aspects. A key issue that needs to be resolved is where the GGAs exert their function in protein sorting. Although all the available evidence points to the TGN as their primary site of action, it is clear that a substantial fraction of the GGAs is associated with peripheral endosomal structures. Some of these structures probably correspond to the large transport carriers that translocate from the TGN, but others seem to be present and stable in the peripheral cytoplasm. Could the GGAs have a role in protein sorting in endosomes? If so, would they be sorting MPRs or other transmembrane cargoes through DXXLL-signal recognition or through other sorting signals? What would be the direction of traffic from this endosomal location — forward to lysosomes or retrograde towards the TGN?

Another unresolved question is why there are three distinct GGAs in humans and mice. Are they largely redundant, as seems to be the case for the two *S. cerevisiae* Ggas, or do they each perform a specialized function? Yet another outstanding issue concerns the relationship between the GGAs and AP-1. Do they cooperate in sorting at the TGN or endosomes? Or do they function at different locations, one at the TGN and the other on endosomes? Crucial to the resolution of these questions will be a better understanding of the function of AP-1, which is unclear at present. Finally, one more avenue of inquiry will involve the study of the GGA-binding partners. A better understanding of these accessory proteins could shed light on the function of the GGAs themselves.

The continuing development of ever more powerful RNA interference methodologies should prove instrumental in answering the outstanding questions about the mammalian GGAs. The combination of genetic and biochemical approaches in yeast should also contribute to the elucidation of the function of the GGAs. If the past 3 years are an indication of a trend, we can expect even more exciting discoveries on the GGAs to be forthcoming.

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Competing interests statement

The author declares that he has no competing financial interests.

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